Chapter 1 NEUROGENESIS IN THE CENTRAL NERVOUS SYSTEM

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Abbreviations: CNS central nervous system, NSC neural stem cells, bHLH basic helix-loop-helix, PNS peripheral nervous system, EGF epidermal growth factor, NIC Notch intracellular domain, DSL Delta/Serrate/lag-2, CSL CBF-1/Suppressor of hairless/Lag-1, pRb retinoblastoma protein, Wg Wingless, Hh Hedgehog, Shh Sonic Hedgehog, BMP bone morphogenetic protein, LEF/TCF lymphocyte enhancer factor/T-cell factor, VZ ventricular zone, SVZ subventricular zone, TGF-α/β transforming growth factor α/β, EGFR epidermal growth factor receptor, FGF fibroblast growth factors, FGFR fibroblast growth factor receptor, HS heparan sulfate proteoglycans, MHB mid-hindbrain boundary, RA retinoic acid, ORN olfactory receptor neurons, PDGF platelet-derived growth factor, BDNF brain-derived neurotrophic factor, NGF nerve growth factors, CNTF ciliary neurotrophic factor, LIF leukocyte inhibitory factor, GH growth hormone, BrdU bromodeoxyuridine, CDK cyclin dependent kinase, CKI CDK inhibitors, NB neuroblasts, GMC ganglion mother cell, aPKC atypical Protein Kinase C, Pins partner of Inscuteable, PON partner of Numb,

Section 1.1 Introduction

Neurons, astrocytes and oligodendrocytes of the central nervous system (CNS) arise from a common pool of proliferating multipotent progenitor cells also called neural stem cells (NSC). In the neural tube, these cells are arranged in a polarized epithelium, the neuroepithelium, also referred to as the ventricular zone (VZ, the layer facing the lumen of the neural tube) (Fig. 1). Neurons are the first cell type to be born from these neural progenitors, with gliogenesis following neurogenesis. In addition to this temporal order there is also spatial order, with neurogenesis proceeding in a rostral-to-caudal direction from the hindbrain to the spinal cord and in a caudal-to-rostral direction from the midbrain to the forebrain. The production of neurons requires the complex integration of environmental and intrinsic cues at the cellular level to control the balance between proliferation, differentiation, and survival.

Upon differentiation signals, which may originate extracellularly as well as intracellularly, NSC generate neurons. They exit the cell cycle (the postmitotic state being a common characteristic of neurons), migrate to the appropriate neuronal layer, and change their gene expression pattern. This early step of neuronal differentiation is then followed by further steps of neuronal maturation, including neurite outgrowth, axon pathfinding, synapse formation, etc.

The neuronal determination of a progenitor cell (its selection from a pool of NSC), constitutes a generic aspect of neurogenesis that can be applied to all types of neurons. However, one should remember that the NSC, and their progeny, are under strict spatio-temporal cues that influence the identity of the resulting neurons. Generic differentiation and neuronal subtype specification are therefore tightly linked and can hardly be dissociated. This chapter will address only generic aspects of neurogenesis. The principal focus will concern mammalian neurogenesis but, whenever required, data from non-mammalian model species will be added. Indeed, the fundamental studies in the fly have

established most of the present concepts about neurogenesis, and the developmental principles are strikingly conserved between species.

The first part of this chapter will discuss the major intracellular players and the complex intrinsic regulation involved in the neuronal determination of progenitors. Then, the most important extracellular factors that influence neurogenesis, and their respective signaling pathways, will be reviewed. The last two parts of this chapter will specifically address the cellular mechanisms underlying the switch of progenitors to neurogenesis. One concerns the role of the length of the progenitor cell cycle on the determination process. The other concerns asymmetric cell division of neural progenitors, which has been pioneered in the fly and for which there is increasing evidence also with regard to mammalian neurogenesis.

Section 1.2 Intrinsic regulation of neurogenesis

In *Drosophila*, like in mammals, NSC are arranged in an epithelium. When neurogenesis starts, only a fraction of these progenitors is instructed to become neurons. This primary step in neurogenesis is crucial, first, to avoid the complete loss of the progenitors, and second, to allow time-dependent factors to influence cell fate. Consequently, the decision to become a neuron is strongly regulated at the cellular level. As neurogenesis begins, positive regulators instructing neuronal differentiation are switched on, and are counteracted by factors involved in the maintenance of the undifferentiated, proliferative state. The balance between inducing and repressing factors determines the onset and extent of neurogenesis.

Neurogenesis is a process that is highly conserved between invertebrates and vertebrates. Orthologs of most of the molecular players involved, both factors promoting and inhibiting neuronal differentiation, have been found in the various species analyzed [1-3].

Section 1.2.1 Neuronal Determination

Section 1.2.1.1 Proneural genes

The major intrinsic regulators of neurogenesis, the proneural genes, were first cloned in *Drosophila* in the late 1970s [4]. Homologs to these genes, the *Drosophila achaete-scute* complex and *atonal*, were then found in vertebrates [1, 5, 6]. Their first characterization, through the analysis of different *Drosophila* mutants, demonstrated their requirement during neurogenesis in the peripheral nervous system (PNS). Loss of function mutants were found to exhibit a loss of external sense organs, while gain of function mutations induce supernumerary sensory bristles [7].

Biochemical characterization and expression

The proneural genes code for transcription factors with a characteristic basic helix-loop-helix (bHLH) domain; the basic domain is involved in DNA binding and the HLH

domain in protein-protein interactions (homo- or hetero-dimerization with other HLH proteins) [8]. Proneural genes are neural-specific transcriptional activators. Upon heterodimerization with ubiquitously expressed bHLH factors (daughterless in *Drosophila* and E2A or E2-2 in vertebrates), their binding to the E-box consensus sequence leads to gene transactivation [9]. Proneural genes in vertebrates are classified according to their *Drosophila* counterparts: achaete-scute complex homolog-like (Ascl), atonal homolog (atoh) and, in addition, the neurogenins.

As observed in *Drosophila*, the expression domains of the proneural genes in vertebrates delimit a competent region where neurogenesis can take place. This characteristic expression in the neuroepithelium is in accordance with an early function in the selection of the future neuron.

Gain- and loss-of-function phenotypes in vertebrates

Gain-of-function experiments (ectopic or over-expression) with proneural genes in vertebrates induce, like in *Drosophila*, supernumerary neurons, in accordance with their expected neurogenic activity [10-12]. These results strongly support the inherent ability of these transcription factors to promote neuronal differentiation of proliferating cells.

In addition, proneural genes are also required during the neuronal differentiation process. Indeed, individual gene inactivation for different proneural genes in mouse embryos triggers a loss of some specific neuronal progenitors [13-16].

Effects induced by proneural activity

So far, the direct target genes of the proneural bHLH transcription factors are poorly characterized. Despite the description of the consensus DNA-binding motif, little is known about the DNA specificity of each proneural gene.

The proneural genes are able to promote neuronal differentiation by integrating different aspects of neuronal development. First, they are involved in the cell cycle withdrawal of the progenitor cells. This is achieved indirectly by the release of lateral inhibition (via the activation of *delta*), which maintains the proliferative state (see below). More directly, the expression of different proneural genes *in vitro* has been shown to upregulate cell

cycle inhibitors [17]. Second, they trigger the sequence of events leading to neuronal differentiation. The proneural genes are able to induce a cascade of transcriptional regulators (mostly from the bHLH class) critically required for the proper neuronal differentiation (see below). Finally, proneural genes influence neural fate determination of the progenitor cells by repressing non-neuronal fate. Notably, they can bias the neuronal versus glial choice; various studies show the active role of the proneural genes in the repression of glial differentiation [18-20]. Concerning this last point, it is remarkable that the expression of proneural genes is nevertheless observed during glial differentiation [21-23]. This may suggest that the neuronal potential of the proneural factor changes with time, or that neural progenitors become unresponsive with time to neuronal identity cues.

It is worth mentioning that proneural genes also play a pivotal role in the identity of the neurons produced from their expression domains, probably by interacting with positional information cues that are present throughout the neural tube [6].

Section 1.2.1.2 Regulation of proneural activity

As described above, neurogenesis is highly dependent on proneural activity. A tight control of the proneural genes is therefore a prerequisite for the proper generation of neurons. The regulation of proneural activity occurs both at the transcriptional and protein level.

Lateral inhibition

Proneural activity is principally regulated by a cell-cell signaling pathway taking place in the neuroectoderm between progenitor cells. This pathway involves the two transmembrane proteins Delta (ligand) and Notch (receptor), which are important cell fate determinants in numerous developmental systems [2, 24]. In *Drosophila*, the Delta-Notch pathway is the key for the lateral inhibition process involved in the selection of a neuronal precursor cell. The term "lateral inhibition" refers to the negative cross-

regulation between neighboring cells that is required to establish the precise pattern of neurons in the fly, or to maintain the pool of progenitors in vertebrates (Fig. 2).

Extensively studied during the development of the fly sense organs, lateral inhibition refers to the process in which one cell is selected from a group of equivalent cells. The expression of a proneural gene (at a low level) defines this equivalence group (or proneural cluster). In these cells, Notch and Delta are also present, each cell having the capacity to send a signal via Delta, but also to receive a signal via Notch. Downstream of Notch signaling are transcriptional repressors that can silence the proneural genes such that the activation of Notch impairs neuronal differentiation. Importantly, the proneural genes induce the upregulation of *Delta*, then activation of Notch in the adjacent cells triggers proneural repression and *Delta* down-regulation, thereby generating a negative feedback loop on the signaling pathway. At equilibrium, signals sent and received by each cell would lead to a mutual inhibition. By inducing an imbalance in the signaling, the auto-amplification of the signal due to the negative feedback on the signaling cascade would induce an all or none response (progenitor fate versus neuronal precursor fate). For example, an increase in proneural activity in one cell (cell A) would induce Delta and activate Notch in the surrounding cells. In response, the proneural genes would decrease and thus *Delta* expression would be reduced in the neighbors. As a consequence, cell A would amplify its own proneural and Delta expression while repressing their expression in the surrounding cells (Fig. 2).

Delta-Notch pathway

Cell biology of Delta-Notch signaling is very complex: both ligand and receptor are tightly regulated [25], including glycosylation [26], ubiquitination [27], endocytosis [28] and proteolysis [29]. Notch and Delta are one-pass transmembrane proteins rich in epidermal growth factor (EGF) repeats. During biosynthetic transport to the cell surface, Notch undergoes a first constitutive cleavage in its ectodomain by the furin protease in the trans-Golgi network. The two proteolytic fragments remain associated and form an active heterodimeric Notch receptor presented at the plasma membrane. Upon Delta-Notch heterodimerization (via the EGF repeats), Notch is subject to additional proteolytic cleavages leading to its activation. Binding of Notch ligands (Delta or Jagged, DSL)

activates extracellular proteases that cleave the extracellular part of Notch and render the C-terminal domain (still attached to the membrane) susceptible to gamma-secretase (presenilin and nicastrin are part of the γ-secretase complex). This last cleavage leads to the release of the Notch intracellular domain (NIC), which enters the nucleus. NIC then forms an activator complex by recruiting the CBF-1/Suppressor of hairless/Lag-1 (CSL) transcription factors and the protein mastermind (Fig. 3A).

In the absence of NIC, CSL represses its transcriptional targets. Notch activation leads to the formation of a transcriptional activator complex (NIC/CSL), which directly transactivates *Hes* genes promoters. *Hes* expression is therefore a powerful read-out of Notch activity.

Hes genes encode transcription factors of the bHLH class with repressor activity also called negative bHLH. They are able to heterodimerize via their C-terminal WRPW domain with the co-repressor Groucho/TLE (*Drosophila*/vertebrates) and to repress target genes [30]. The principal targets of Hes factors are the proneural genes. In addition to transcriptional repression of the proneural genes, Hes factors can also block the proneural proteins by forming non-active heterodimers with the proneural bHLH proteins, thereby inhibiting their activity [31] (Fig. 3B).

Notch activity associated with CSL factors is known as the canonical Notch pathway. Some studies, however, suggest the existence of other Notch pathways, which are independent of CSL activity [32, 33].

Notch signaling during mammalian neurogenesis

In mammals, all four *Notch* genes are expressed in the proliferative region at specific stages of nervous system development. Manipulations of *Notch* expression or of genes in the Delta-Notch pathway show neuronal phenotypes.

Notch1 inactivation results in early lethality. Nevertheless, it is possible to determine that in the absence of *Notch1,* there is a premature expression of proneuronal genes and a premature generation of neurons [34]. Consistent with the previous results, the conditional inactivation of *Notch1* in the midbrain also shows neurogenesis defects [35].

In complement to the inactivation experiments, overexpression of NIC in mice decreases neuronal production [36].

In accordance with the function of the Notch pathway during neurogenesis, manipulations of the different proteins in the signaling pathway also induce neuronal phenotypes. Impairing Notch processing using a presenilin mutant [37] or inactivating *Hes1* and *Hes5* (the principal target genes of the Notch pathway during neurogenesis) produces neurogenesis defects similar to the one observed in the *Notch* inactivation mutants. In addition, *Hes* genes, when overexpressed, can repress neuronal differentiation [38] while their inactivation causes premature neuronal differentiation [39]. The general effects on neurogenesis described above have also been associated to defects in the expression of the crucial regulators of neurogenesis, the proneural genes.

To conclude, lateral inhibition, via its principal effectors, the Hes repressors, is critically required to regulate neuronal determination by tightly controlling of the proneural activity.

Section 1.2.1.3 Proliferative determinants

Proliferation of neuroepithelial cells is not only regulated by the Delta-Notch signaling pathway but also by various factors expressed in the progenitors themselves. Among these proliferative factors, the genes of the Id family are one class of interesting transcription factors that act probably again by tuning the proneural activity.

Id proteins encoded by four *Id* genes in vertebrates (homolog to *Drosophila extramacrochaete*) are transcription factors with an HLH domain but no DNA binding domain. They are thus able to form heterodimers with bHLH proteins via the HLH domain. Because they lack the basic DNA-binding domain, the heterodimer generated is unable to bind DNA and therefore, act as a dominant-negative complex [40].

In the neural tube, *Id* gene expression is specifically restricted to the proliferative region, and inversely correlated with neurogenesis, suggesting a role in proliferation of NSC. Id proteins are involved in the control of cell differentiation and cell cycle progression in different cell lineages. During neurogenesis, they repress proneural activity and regulate

the cell cycle. Mice inactivation studies show a size reduction of the neural tube associated with premature differentiation, in accordance with a role in the inhibition of differentiation [41]. Moreover, overexpression of Id2 induces an overgrowth of the cortex in addition to a block of cortical differentiation [42]. Apart from the block of proneural activity that would be associated with the natural dominant effect of Id on bHLH factors, it has also been shown that the effect on cortical differentiation could be reverted by coexpressing pRb, the retinoblastoma protein, suggesting a direct link with cell cycle regulation.

Neurogenesis, and more precisely the neuronal determination as the first chronological event leading to neuronal generation, is strongly dependent on proneural gene expression and function. As shown in the above section, various levels of regulation are required to shape proneural activity.

Section 1.2.2 Neuronal differentiation

Once the neural progenitor has given rise to a neuron, a cascade of differentiation genes is turned on. The proneural genes, as transcriptional activators, are able to start this neuronal differentiation program that is characterized by the sequential activation of different genes (mostly transcription factors) essential to implement the neuronal phenotype.

One of the first genes transiently turned on by the proneural factors is *Delta* (as described above), which is followed by the expression of other members of the *bHLH* family of transcription factors whose expression is specific to the neural tissue. Among these genes, NSCL1, NeuroD and Math3 are transiently expressed in the young postmitotic neuron that is en route to the neuronal layer. Depending on the region of the nervous system observed, different sequences of gene expression are activated in the young neurons. For example NeuroM is turned on before NeuroD in the spinal cord, but after it in the dorsal root ganglia [43].

The ectopic expression of such bHLH genes, like the proneural bHLH genes, is characterized by a neurogenic activity. However, their expression pattern is not consistent with an early role in neurogenesis and gain and loss-of-function analyses suggest rather that they have roles in the maintenance of the differentiated state, in cell cycle exit, and even in survival of specific neuronal populations [44-46].

Other transcription factors are also critically involved during the differentiation process. Zinc-finger transcription factors (EBF family, Zic family) seem to be able to coordinate neuronal differentiation and could function to reinforce the identity of committed neurons or to promote neuron survival [47, 48].

Very few of the effector targets of these transcription factors have been identified but one could assume that a lot of important determinants are mobilized to transform the proliferative progenitor to a differentiated cell. Various biological modifications (cell cycle exit, cellular mobility, cell shape remodeling) are involved in this transformation and are still to be characterized.

Various regulators not cited above are also involved in the process of neurogenesis and participate in the overall spatial and temporal patterning of neurogenesis. The gradual acquisition of a generic neuronal identity is dependent upon complex transcriptional regulation as suggested in this section. Each step being highly controlled by different factors necessary to both, maintain the pool of progenitor cells and stimulate neuronal generation.

Section 1.3 Extrinsic regulation of neurogenesis

This chapter will focus only on the effect of different extracellular factors on neurogenesis in the CNS. A review of different signaling factors acting on the balance of proliferation-differentiation is presented here, with the principal aim to demonstrate their function in neurogenesis, either as inhibitors or as activators of differentiation.

Most of the known signaling pathways seem to be involved in the establishment of the nervous system, providing the extracellular cues for the spatio-temporal control of neurogenesis. Some of these factors are known to act in the maintenance of NSC proliferation, which is critical for neurogenesis, either directly stimulating proliferation, promoting survival, or both. Other factors are also actively required to promote neuronal differentiation. In both cases, the deregulation of the pathway associated with these factors often leads to neuronal generation defects.

Section 1.3.1 Wnt factors

The Wnt (Wingless/Int-1) proteins play diverse roles during development, acting on cell proliferation, cell polarity and cell fate determination [49]. Wnt family members stimulate stem cell proliferation in various tissues including the CNS [50-53].

Section 1.3.1.1 Wnt pathway

Members of the Wnt family are secreted signaling glycoproteins related to *Drosophila* Wingless, Wg (reviewed by [54]). Intracellular signaling of the Wnt pathway involves at least three diverse branches. First, the beta-catenin pathway also called canonical Wnt pathway, which activates target genes in the nucleus. Second the planar cell polarity pathway, which involves jun N-terminal kinase (JNK) and cytoskeletal rearrangements. Third, the Wnt/Ca2+ pathway (for a recent update see [55]). The core components of these pathways are the Frizzled seven-pass transmembrane receptors and the single transmembrane co-receptors LRP (low-density lipoprotein receptor related proteins).

Intracellularly, the canonical Wnt pathway leads to the stabilization of cytosolic β-catenin, which is otherwise degraded by a huge protein complex. This complex consists of the casein kinase, the glycogen synthase kinase 3beta (GSK3beta), the scaffold proteins called axin and diversin, and the tumor suppressor gene product APC. This complex triggers phosphorylation of β-catenin and its degradation by the ubiquitin-proteasome pathway. In the presence of Wnt, Dishevelled blocks β-catenin degradation, allowing the stabilized β-catenin to enter the nucleus where it associates with the transcription factors LEF/TCF (lymphocyte enhancer factor/T-cell factor). The complex β-catenin/LEF/TCF activates the Wnt target genes.

Extracellular antagonists of the Wnt signaling pathway have been characterized in different developmental systems as Wnt signal modulators. The antagonists can be divided into two broad classes depending on their mode of action (for review see [56]). Both classes of molecules prevent ligand-receptor interactions, but by different mechanisms. Members of the first class, which include the sFRP (secreted Frizzled-related protein) family, WIF (Wnt inhibitory factor)-1 and Cerberus, bind directly to Wnt proteins. The second class of factors comprises certain members of the Dickkopf (Dkk) family and act by binding to one component of the Wnt receptor complex. The two classes of antagonist inhibit differentially the canonical and non-canonical Wnt pathway, and they can also antagonize each other [56].

Section 1.3.1.2 Propagation of the Wnt signal

Wnt signaling molecules are secreted proteins able to function at a distance from their source of production [57]. During *Drosophila* development Wg can act as a long-range morphogen directly inducing transcriptional targets in a concentration-dependent manner [58, 59]. However, whether these molecules are classical morphogens can be questioned particularly regarding the concentration-dependent responses they induce (for review see [60]). In *Drosophila*, Wnt are proposed to act as long-range signals (20–30 cell diameters away from their site of synthesis [59]). In vertebrates, however, Wnt factors have been shown to bind with high affinity to proteoglycans of the extracellular matrix thereby restricting the likelihood of their diffusion [61].

The mechanism used by Wg to reach its target is still unclear. So far wingless has been proposed to spread by restricted diffusion, cell delivery and planar transcytosis (reviewed [62]). First, Wg carrying vesicles could be inherited during proliferation and transported with the moving cell [63]. Second, Wg carrying vesicles could be actively transported through cells by planar transcytosis [64]. To support the previous hypotheses, vesicles containing the Wg protein have been found in Wg-responsive cells in *Drosophila* embryonic imaginal discs. The restricted diffusion model explains the formation of the unstable Wg gradient on the basolateral surface of the wing imaginal disc epithelium [65]. The restricted diffusion model suggests that Wg spreading is constrained by interactions with cell surface or extracellular matrix. Dynamin-dependent endocytosis has also been involved in the secretion of Wg, but not in Wg spreading in the extracellular space. Endocytosis seems to contribute to the shaping of the gradient by removing extracellular Wg [65]. The Wg morphogen gradient forms by rapid movement of ligand through the extracellular space, and depends on continuous secretion and rapid turnover.

Section 1.3.1.3 Expression and function during neurogenesis

mRNA expression of six Wnt (Wnt1-6) homologs has been analyzed in detail in embryonic and adult mouse tissues [66]. The most prominent member of the family, Wnt1, is expressed along the entire antero-posterior axis of the neural tube in the dorsal midline, and in a belt-like fashion at the midbrain-hindbrain boundary. Wnt7a is expressed in the germinal layer of the embryonic mouse cortex [67], as are several frizzled receptors [68]. Wnt7b is expressed in the cortical plate, particularly by deep layer neurons [69, 70].

Analyses of inactivated mutants for one or more genes of the Wnt signaling pathway tend to corroborate the idea that the Wnt pathway acts in the maintenance of cellular proliferation through its mitogenic action. For example, the midbrain of Wnt1 mutant mice is deleted [71, 72], as well as the hippocampus of Wnt3a mutants [52] and LEF1 mutants [73]. The Wnt1/Wnt3a double mutants show a reduction of the caudal diencephalon, the rostral hindbrain and the cranial and spinal ganglia [74]. In the case of mice lacking Wnt3a, caudo-medial cortical progenitors appear to be specified normally, but then they underproliferate. By mid-gestation, the hippocampus also presents a strong

phenotype, being either absent or constituted by a few residual cells [52]. These findings suggest that Wnt1 and Wnt3a play broad, semi-redundant roles in growth control of the neural progenitors rather than simply specifying regional cell fates as it is broadly documented [75]. Consistent with this view, ectopic expression of Wnt1 in transgenic mice causes an overgrowth of the neural tube without altering the primary patterning of cell identities along the dorso-ventral axis [76].

Wnt factors are not only acting during brain development, but also in spinal cord neurogenesis. In the spinal cord, neurogenesis proceeds in a ventro-dorsal wave, opposed to the dorso-ventral mitogenic gradient. Different Wnt ligands are expressed in the spinal cord. They can be classified depending on their expression and on their mitogenic activity: Wnt1 and Wnt3a, which are restricted to the dorsal midline of the spinal cord, have a mitogenic activity, while more broadly expressed Wnt factors (Wnt3, Wnt4, Wnt7a, Wnt7b) do not. The mitogenic Wnt factors form a dorsal to ventral concentration gradient that correlates with the growth gradient established in the neural tube as it grows; the proliferation rate is highest dorsally while the differentiation rate is highest ventrally [53]. In order to describe the morphogenesis of the spinal cord, a "mitogen gradient model" has been proposed. The predictions from this model are in good correlation with results from mutant analyses. Loss of function of mitogenic Wnt factors like the double mutant Wnt1/Wnt3a leads to a marked reduction in the number of spinal cord neural precursors, with the phenotype being more pronounced in the dorsal part of the spinal cord [74]. Mouse mutants of non-mitogenic Wnts (inactivation of Wnt4 or Wnt7a/Wnt7b) do not show such phenotypes, their neural tubes grow normally [53]. Wnt3 mutant mice die at gastrulation, therefore, it is not possible to analyze the possible effects on spinal cord neurogenesis of these embryos [77].

Section 1.3.1.4 Effectors of the Wnt pathway

As described above, Wnt pathways converge on the β-catenin/LEF-TCF transcriptional complex. Some of the target genes have been characterized and their deregulation confirms that they act in the Wnt pathway. Consistent with the Wnt mitogenic effect, the constitutive activation of β-catenin in mouse neural precursors shows that they re-enter the cell cycle rather than differentiate [78-80]. The expression of stabilized β-catenin in

embryonic or adult mice leads to an expansion of the progenitor pool, a horizontal enlargement of the VZ and a massive folding of the cortex. This dramatic disorganization of the neocortical layering occurs despite the presence of increased apoptosis [78, 79].

Emx2, a homeobox gene, is regulated in the forebrain through LEF/TCF enhancer sites, strongly suggesting that it is a target gene of the Wnt canonical pathway [81]. Interestingly, *Emx2* has been shown to control proliferation and promote symmetric cell divisions and multipotency of cortical progenitors [82, 83]. The overgrowth induced by constitutive β-catenin expression also suggests that progenitor cells are biased toward a symmetric proliferative division, indicating that the activity of β-catenin could be regulated through the regulation of *Emx2*. The stimulation of proliferation induced by β-catenin could also be more direct. In fact, the canonical Wnt signaling pathway has also been shown to regulate some cell cycle regulators. More precisely, in spinal neural progenitors, the β-catenin/TCF complex can promote cell cycle progression and repress cell cycle exit through the transcriptional activation of *cyclin D1* and *cyclin D2* [53].

In addition to its function as a key mediator of the intracellular Wnt pathway, β-catenin interacts with cadherins and cytoskeletal components and is therefore involved in cell adhesion, cell polarity [84, 85] and in mechanisms determining the symmetry of cell division [86]. Mutations or immunogenic inactivation of zebrafish N-cadherins, which bind β-catenin, disturb the integrity of the neuroepithelial VZ [87, 88]. Along this line, the conditional inactivation of β-catenin in mouse from E10.5 onward, triggers a dramatic disorganization of the telencephalic neuroepithelium. This defect is also associated with disruption of interkinetic nuclear migration, loss of adherens junctions, block of neuronal radial migration and a decrease in cell proliferation after E15.5 [89]. In newborns, a premature disassembly of the radial glial scaffold and an increased number of astrocytes are also detected in the cortex [89]

Section 1.3.1.5 Cross-talk between signaling pathways

Neurogenesis in the cortical region of mammals requires a first step of progenitor maturation, which is characterized by the transition of progenitors from the VZ to the subventricular zone (SVZ), where they continue to divide before differentiating. Wnt

ligands, in addition to their mitogenic activity, are involved in this maturation step in a common pathway involving FGF2, Shh and BMP4 [90].

The canonical Wnt pathway may cross talk with the transforming growth factor β (TGF-β)/bone morphogenetic protein (BMP) signaling or other cytokine signaling through the mitogen-activated protein kinases (MAPK) pathway [91]. It seems actually that some of the MAPK can counteract Wnt signaling through phosphorylation of the β-catenin/LEF-TCF complex [91]. A similar negative regulation between the Wnt and TGF-β/BMP pathway has also been described in *Drosophila*. In this case, decapentaplegic (BMP homolog) triggers the repression of *Wg* by inactivation of Hh [92].

Section 1.3.2 Hedgehog factors

The Sonic Hedgehog (Shh) signaling pathway is highly conserved and involved in various developmental processes. The most studied function of this molecule concerns the establishment of the dorso-ventral patterning in the CNS. In patterning, Shh acts as a classical morphogen; different cellular fates can be induced by Shh depending on the concentration of Shh received that is thus dependent on the distance of the Shh-sensitive cell from the source of Shh. Besides its role in patterning, Shh also plays a role in proliferation of neural precursors and axonal growth.

Section 1.3.2.1 Shh pathway

Shh is a member of the Hh family of signaling molecules (reviewed in [93]). Shh is a secreted molecule that is also found at the cell surface. It acts as a morphogen, and triggers different cell fates in a concentration-dependent manner. The protein follows different maturation steps during its synthesis. First, an autocatalytic cleavage releases the N-terminal active peptide that is then modified by addition of a cholesterol molecule in the C-terminus and a palmitoyl group in the N-terminus [94]. Different hypotheses have already been proposed to explain the mode of diffusion of Shh, however, it is still unclear how it works as a long-range signal [95]. It has recently been shown that the megalin endocytic-receptor can also bind Shh suggesting that endocytosis could be part of the mechanism of Shh diffusion in tissue [96, 97].

Shh binds its receptor Patched (twelve-pass transmembrane protein), which releases the Smoothened seven-pass transmembrane protein and triggers the activation of the Gli zinc finger transcription factors [94].

Section 1.3.2.2 Expression and function during neurogenesis

Shh is produced by two ventral midline signaling centers: the notochord, the axial mesoderm underlying the neural tube, and the floor plate, a specialized population of cells at the ventral midline of the neural tube [98]. As development proceeds, Shh appears in more rostral locations: in the zona limitans interthalamica, and in the medial ganglionic eminence in the basal telencephalon. During late development and in adulthood Shh is found in the cerebellar cortex and the optic tectum [99, 100]. Shh is also expressed by differentiated cells of the mouse cortex, in a layer specific manner [101]. The Gli transcription factors are also expressed in the CNS; they are notably found in progenitor populations of the cortex and of the spinal cord [101, 102].

The cerebellar cortex originates from two distinct germinal layers, a typical VZ and an external germinal layer. The latter exists only transiently and contains granule cell progenitors. After clonal expansion, the granule cells exit the cell cycle and migrate through the Purkinje cell layer to their final location. Shh is expressed in migrating and settled Purkinje neurons and acts as a potent mitogen to expand the granule cell progenitor pool [103-105]. Granule cell maturation and migration happens therefore in a Shh rich environment, which is in contradiction with Shh being a mitogenic factor for the granule cells. In this context, modulation of Shh response seems to be dependent on the extracellular matrix. Laminin glycoproteins present in the proliferative region stimulate Shh induced proliferation, while vitronectins, encountered when the cells migrate, down-regulate the mitogenic response to Shh [106, 107]. The vitronectin effect could be mediated by its ability to phosphorylate the cAMP responsive element binding protein (CREB) that is alone able to trigger the differentiation of the granule cells despite the presence of Shh [106].

Manipulations of Shh expression have been used to define the function of the pathway during neurogenesis and have shown that Shh activity can regulate patterning,

proliferation and fate determination. Ectopic Shh expression in the mouse dorsal spinal cord increases the proliferation rate of precursors at early stages [108]. In the same study, Shh responsive cells later in development, were found to be postmitotic, suggesting that the mitogenic competence of Shh varies with time [108]. Additionally, and still in line with the mitogenic effect of Shh, misexpression of Shh in the embryonic telencephalon leads not only to a ventralization of the tissue [109] and the appearance of supernumerary oligodendrocytes [110] but also to an abnormal proliferation of the neural precursors and pronounced hypertrophy of the telencephalic region [101, 111]. In the postnatal telencephalon, Shh signaling both promotes proliferation and maintains the neural progenitor state [112]. In adult brain regions, it has been suggested that it is involved in maintaining stem cell niches [112].

In general agreement with the gain-of-function phenotypes, the Shh null mutants have defects in dorso-ventral patterning, ventral fate specification and cell proliferation. In these mutants the telencephalon is dysmorphic, strongly reduced in size (up to 90%) and appears as a single fused vesicle [113, 114]. Oligodendrocyte differentiation markers are also missing in Shh mutant mice, in accordance with its function in the specification of ventral identity [115].

The diverse results presented so far show that Shh action is crucial to build the vertebrate brain through the regulation of stem cell numbers, the control of precursor proliferation and the organization of cellular identity [101, 103-105, 116]. Overall or local changes in Shh levels or its reception, may have contributed to the evolution of sizes and shapes of the brain such as expansion of the primate neocortex, or the tectum in birds, and the cerebellum of electrosensitive fish. The price for such plasticity may be tumorigenesis. Many cancers arise from constitutive Shh signaling in various tissues, including the brain (reviewed by [117, 118]).

Section 1.3.2.3 Eye differentiation as a model for Shh activity

After formation of the optic cup, multipotent retinal precursors give rise to all major cell types in the retina [119]. Neuronal differentiation is usually initiated in the central retina and subsequently spreads into the periphery [120]. Shh, the related tiggywinkle and

desert hh, the Shh receptors, Patched 1 and 2 are expressed in differentiating eye cells [121, 122]. The production of Shh in the ventral diencephalic midline accounts for the early patterning in vertebrates whereas endogenously expressed members of the Shh signaling pathway control both proliferation and differentiation of retinal precursor cells [121-123].

In zebrafish, neuronal differentiation is initiated in the ventro-nasal rather than the central retina [124-127]. Shh secreted by the differentiated retinal ganglion cells drives the wave of neurogenesis from the centre to the periphery of the retina. The propagation of the neurogenic wave involves the induction of its own expression in the uncommitted cells and the activation of the Ras-MAP kinase pathway [123].

Similarly to what happens in vertebrates, a neurogenic wave sweeps across the *Drosophila* eye imaginal disc and generates ommatidia behind the progressing morphogenetic furrow [128, 129]. Hh initiates neurogenesis in adjacent undifferentiated cells through induction of the expression of *atonal*, a proneural bHLH transcription factor. Newly differentiated neurons in turn secrete Hh that initiates further neuronal differentiation and progression of the morphogenetic furrow [130].

Section 1.3.2.4 Cross-talk between signaling pathways

The exposure of cells to various signaling factors can be either sequential or concomitant. An example of the first concerns the generation of different cell types through the sequential action of Shh and BMP. During development of the cerebral cortex, gamma-aminobutyric acid (GABA)ergic neurons and oligodendrocytes are generated from a common neural stem cell population located in the VZ of the ventral forebrain. Most of them reach their final position after tangential migration. During their maturation, these progenitors therefore experience different signaling cues dependent upon their position. First, ventral Shh restricts the progenitors to GABAergic neuronal and oligodendrocytic programs by the induction of bHLH transcription factors, like Olig2 and Mash1 [131]. Subsequent exposure to different levels of BMP determines the neuronal versus glial choice. Neuronal differentiation is implemented by activation of the BMP pathway, while the presence of BMP antagonist (noggin) allows oligodendrocyte differentiation [132].

In the mouse neocortex, Shh and EGF signaling may synergize through the regulation of the EGF receptor (EGFR) to control precursor proliferation [133]. A negative interaction between the Shh pathway and BMP pathways seems also to occur at the level of the Gli activity [134]. It has been shown that the effector of the BMP pathway, the smad protein, can bind the Gli3 thereby providing the means to counteract Shh activity. The Gli proteins seem also to be modulated by some components of the Wnt pathway [135].

Section 1.3.3 Fibroblast growth factors

Fibroblast growth factors (FGF) are widely involved in developmental processes and are present in almost all mammalian tissues. In the nervous system, FGF are not only involved in early neurogenesis, but also in axon growth, neuroprotection, and synaptic plasticity.

Section 1.3.3.1 Ligands, receptors and expression during neurogenesis

FGF ligands are monomeric molecules that form multimers under association with heparan sulfate proteglycans (HS). The cluster of FGF ligands triggers FGF receptor tyrosine kinases (FGFR) activation [136, 137]. Activation of the receptor by autophosphorylation in turn allows the transient assembly of multiple intracellular signaling complexes.

Ten out of 23 FGF family members are expressed in the brain, and 4 receptors (FGFR) were identified so far. The analysis of the expression pattern of different FGF shows that some of them are expressed in relevant brain regions during neurogenesis suggestive of an active role during this process [138]. Among all the FGF, FGF1 (acidic FGF), FGF2 (basic FGF), FGF6 and FGF7 are expressed in the proliferating mouse neuroepithelium [138, 139]. Moreover, FGFR isoforms are also detected in the VZ at early developmental stages showing that, indeed, FGF signaling can be integrated at the level of neural progenitors [140]. In addition, heparin and HS, which are thought to play a role in the ligand presentation, are secreted by neuroepithelial cells. Looking at various developmental times, different glycosylation states of HS are detected and have been associated to a modulation of HS affinity for various FGF. FGF1 and FGF2, which are

expressed sequentially in the brain when neurogenesis starts, could therefore be modulated by the HS form present in the tissue [138].

Section 1.3.3.2 Function during neurogenesis

In vitro, FGF2, like EGF is known and widely used to stimulate the mitotic activity of various stem cells dissociated from embryonic and adult mouse brain regions. Because both neurons and oligodendrocytes can be obtained from NSC stimulated by FGF2, it is thought that this action is not neuronal-specific [138, 141]. However, it has also been suggested that FGF2 can stimulate preferentially the proliferation of neuronal progenitors coming from spinal cord progenitor cultures [142]. A proper analysis of FGF2 dose-effect has also demonstrated that low doses of FGF2 could contribute to enrich the culture in neuronal-committed precursors [140]. Indeed, cortical progenitor cells in culture are able to differentiate preferentially into neurons in the presence of FGF2. This effect seems to be mediated by the MEK-C/EBP-ERK pathway, as FGF2 is able to stimulate the phosphorylation of MEK kinases in these NSC cultures. Additionally, blocking or enhancing ERK phosphorylation (a MEK substrate) affects neurogenesis promoted by FGF2 on behalf of gliogenesis [143].

A mitogenic effect promoted by FGF has clearly been shown *in vitro*, raising the question of the relevance of this effect *in vivo* and of its ability to increase the pool of neuronal progenitors. The presence of the components of the signaling pathway in the appropriate time during development are already bringing some evidence for such an activity *in vivo*. Mice lacking *FGF2* show a reduction in neuronal density in the neocortex suggesting a role for FGF2 during corticogenesis [144]. In the same line of evidence, injection of anti-FGF2 antibodies decreases the number of neural cells produced while intra-luminal injection of FGF2 generated additional neurons and glial cells [145]. Together, these results show the *in vivo* mitogenic activity of FGF2 and its importance to stimulate the proliferative capacity of neural progenitors and, as a consequence, to regulate the number of neurons produced in the cortical region. A similar effect also seems to be triggered by FGF8 in another specific domain of the brain, again arguing for the involvement of FGF in the regional modulation of cellular growth. FGF8 is expressed in the midbrain-hindbrain junction (MHB) where it acts as a mediator of the organizer activity that is

regulating the proper patterning of the adjacent midbrain region. Besides its patterning function, FGF8 also seems to regulate the overall growth of the midbrain region [146]. At the MHB, additional FGF are also expressed, FGF18 and FGF17, and inactivation of *Fgf17* leads to midbrain tissue loss due to a decrease of precursor cell proliferation [147].

Section 1.3.3.3 Cross-talk between signaling pathways

The FGF pathway interacts with other signaling pathways to integrate intrinsic information or other important cues. Interactions between the Notch pathway and the growth factors FGF1 and FGF2 have been described *in vitro* while examining the mechanisms of action of FGF on neuronal differentiation [148]. E10 neural progenitor cells from the forebrain, sensitive to the mitogen activity of FGF (FGF1, FGF2 or FGF8 for instance) increase *Notch1* expression and decrease *Delta* expression. Moreover, by interfering with Notch activity (blocking or activating) the inhibition of neuronal production normally induced by FGF is perturbed. As both pathways are required to block neurogenesis, this study suggests that at least a part of FGF activity is mediated via the Notch pathway.

Two recent studies from the laboratory of K. Storey provide a nice example of exogenous influences on neurogenesis, and at the same time reveal the opposite interactions of FGF and retinoic acid (RA) pathways on neurogenesis [149, 150]. The spinal cord is surrounded by paraxial mesoderm. As development proceeds, both tissues appear to differentiate concomitantly in a rostro-caudal sequence: the spinal cord generates neurons and the mesoderm forms somites. The authors have shown that the paraxial mesoderm is able to influence neuronal differentiation in the adjacent neuroectoderm. The undifferentiated mesoderm, or presomitic mesoderm expresses FGF while the somites (differentiated mesoderm) express RA. On the other hand, RA receptors are expressed in the spinal cord at the level of the somites in the region where neurogenesis is starting. The results suggest that FGF signaling from the presomitic mesoderm (but also from the caudal neural plate) is involved in the maintenance of the undifferentiated state in the neuroectoderm and that a RA signal coming from the somites can promote neuronal differentiation. The effects of RA are partly due to its ability to down-regulate *FGF* expression (*Fgf8*), but a more active process also seems to be involved, as repressing the

FGF pathway alone is not sufficient to promote neurogenesis. FGF8 can also regulate RA production thereby providing a cross-regulation feedback that could be essential to coordinate both neurogenesis and somitogenesis.

All together, the *in vivo* and *in vitro* results about FGF function point to a role in the regulation of the proliferative pool of progenitors, providing a means to regulate the timing of neurogenesis and the number of neural cells produced.

Section 1.3.4 Transforming growth factors-α, neuregulins and epidermal growth factors

Although they are not as widely expressed as the FGF family of ligands and receptors, TGF-α and EGF are also expressed in patterns that are suggestive of a role in regulating proliferation of precursor populations in the developing and adult nervous system.

Section 1.3.4.1 Ligands, receptors and expression during neurogenesis

TGF-α, EGF and neuregulins signal via a common receptor, EGFR a tyrosine kinase receptor encoded by the *ErbB* gene. The proteins alternately referred to as neuregulins, neu differentiation factors (NDF), glial growth factor (GGF), and acetylcholine receptor–inducing activity (ARIA) are encoded by a single differentially spliced gene. Neuregulin receptors are encoded by the *ErbB-2-4* genes (reviewed in [151]). EGF, like FGF, is a monomeric ligand that bind to EGFR and thereby triggers receptor dimerization and activation.

TGF-α is expressed in the proliferating cells of the developing rat basal ganglia by E13, in the germinal zone of the midbrain by E15, and in the VZ of the medial ganglionic eminence by E17. EGFR mRNA is found in the germinal zone of the midbrain and the external granule layer of the cerebellum by E15 [152]. Postnatally, EGFR continues to be expressed in regions undergoing active neurogenesis including the cerebellar granule layer and the SVZ [153]. EGFR is also expressed, as observed by immunohistochemistry, in the granule layer and in proliferating cells of the dentate gyrus [154].

Section 1.3.4.2 Function during neurogenesis

A lot of evidence from *in vitro* studies demonstrates that EGFR ligands regulate proliferation of distinct precursor populations. For example, precursors from fetal rodent striatum that are expanded using EGF as a mitogen stay multipotent and can give rise to the three major cell types of the CNS: neurons, astrocytes, and oligodendrocytes [155, 156]. Similarly, stem cells isolated from the adult SVZ proliferate in response to EGF [157-159]. Transitory amplification of the cells of the adult SVZ shows that these cells retain stem cell competence under the influence of EGF signaling [160]. The same mitogenic effect is also observed using TGF-α. For example, retinal progenitor cells from embryonic and postnatal rats maintained as explants or in monolayer culture proliferate in response to TGF-α dependent on the maturation stage of the cells [159, 161, 162]. Finally, EGF is a mitogen for dissociated precursors from postnatal rat olfactory epithelium [163, 164].

In contrast to the proliferative effects of FGF2 on early VZ progenitors, EGF is a potent mitogen for the late multipotent progenitors of the embryonic and adult SVZ [156, 165]. The transition from VZ to SVZ in the mouse cerebral cortex is correlated with the upregulation of EGFR [152, 153], which confers mitotic responsiveness to EGF family ligands [166]. The EGF-responsive population represents a subset of SVZ cells and is itself heterogeneous, including stem cells and more restricted precursors. Maturation to an EGF-responsive state requires positive and negative regulators such as the BMP or FGF2 [167]. In addition, TGF-α also seems to have a mitogenic function. Adult mice with a targeted deletion in the *TGF-α* gene show diminished proliferation of precursors within the SVZ, consistent with a proliferative function during neurogenesis [168]. Moreover, mice lacking functional EGFR have defects in cortical neurogenesis, which can be associated to a role of EGFR ligands in proliferation, differentiation, migration or survival of neural precursor populations [169].

Besides their role in proliferation, EGFR ligands appear to play a role in regulating differentiation of precursor populations. Rat retinal progenitor cells expressing exogenous EGFR *in vivo* following infection with a retrovirus encoding the human EGFR differentiate preferentially into Mueller glial cells [162]. In explant cultures, this glial

differentiation occurs at the expense of rod photoreceptor cell differentiation, suggesting that activation of EGFR in retinal precursors regulate such lineage decisions. Long-term administration of EGF to the lateral ventricles of adult rats does not appear to induce the generation of dentate gyrus or olfactory bulb granule neurons, and instead induces generation of astrocytes within the SVZ [159]. Evidence also suggests roles for other members of the EGF family in regulating lineage decisions during neural development. Targeted deletion of neuregulin [170-172] or of the genes encoding ErbB2 [173], ErbB3 [174], or ErbB4 [175] resulted in mice having profound defects in CNS or PNS populations of neurons or glia. Among the most prominent defects described in these studies, the dramatic reduction in Schwann cells in mice lacking ErbB3 indicates a role for neuregulin signaling in generation of this lineage from the neural crest.

The different ligands signaling through the EGFR seem to have different roles in neurogenesis. On one hand they were shown to stimulate proliferation and used as mitogen factors, on the other they seem to be involved in the gliogenic lineage decision. These two aspects of EGF signaling which are in apparent contradiction may be reconciled by considering the chronology of neuronal and glial differentiation.

Section 1.3.5 Transforming growth factors-β

Members of this family play critical roles in regulating developmental processes, so it is not surprising that they function during the development of the nervous system. However, identifying their precise roles in regulating the generation of neuronal populations has been a difficult problem.

Section 1.3.5.1 Ligands, receptors and expression during neurogenesis

The TGF-β superfamily of ligands is extremely large, with over 40 members identified in different organisms from *Drosophila* to mammals. The superfamily includes TGF-β members, activins and BMP. TGF-β members are secreted peptide growth factors forming dimers. They exert their effects through a class of heterodimeric receptors with serine-threonine protein kinase activity [176]. Once activated, the receptors phosphorylate the Smad proteins (Smad2 and -3 are phosphorylated by TGF-β and

activin family members while Smad1, -5 and -8 are phosphorylated by BMP). This triggers the formation of a Smad complex, which enters the nucleus, recruits cofactors and regulates target genes.

TGF-β1, -β2, and –β3 are expressed in the developing rodent brain and spinal cord [177-180], but, in contrast to the expression patterns of TGF-α and FGF family members, they are highly expressed in regions of neuronal differentiation and not in proliferative zones. In the postnatal rat brain, TGF-β2 is also expressed in the hippocampus, dentate gyrus, and cerebellum [181].

Section 1.3.5.2 Function in neurogenesis

The expression pattern of TGF-β argues against a general role in early progenitor proliferation. However, some TGF-β members have been shown to regulate proliferation in certain neural populations. TGF-β3 is mitogenic for rat retinal precursors *in vitro* and enhances the mitogenic effects of EGF and acidic FGF [182]. In the same culture, the presence of TGF-β3 increases the number of retinal neurons, suggesting that it acts on the precursors of retinal neurons [182]. This latest result and others are suggestive of a role in the commitment of the progenitor or in the promotion of differentiation. For example, despite the presence of the mitogenic EGF, TGF-β2 can induce the generation of olfactory neurons *in vitro* from olfactory epithelium cultures [163]. In addition, TGF-β2 inhibits the proliferation of cerebellar precursors [181, 183], although this effect can be modulated with the culture conditions [183]. TGF-β is also an anti-proliferative signal for pluripotent neural crest cells and for committed melanogenic cells; the TGF-β-mediated anti-proliferative activity dominates over the FGF-2/neurotrophin-mediated mitogenic signal, and enhances sensory and adrenergic neurogenesis [184].

Section 1.3.5.3 Cross-talk between signaling pathways

Neurogenesis continues throughout adult life in the mammalian olfactory epithelium. This is a very dynamic process involving proliferation, differentiation and cell death. It is highly likely that not only different autocrine, but also paracrine signals, regulate it.

Numerous *in vitro* studies about the olfactory receptor neurons (ORN) suggest that TGF-β promotes the maturation and/or differentiation of olfactory progenitors. However, the physiological relevance of these effects as well as the exact mechanisms of action can be questioned. Nonetheless, there is emerging evidence that FGF2, TGFβ-2 and platelet-derived growth factor (PDGF) act sequentially on precursor cells and immature neurons during adult olfactory epithelium neurogenesis [185].

Both mature and immature ORN express the TGF-β type II receptor (TGFβ-RII), suggesting that these cells could effectively respond to a TGF-β signal [186]. In the olfactory epithelium of TGF-α overexpressing transgenic mice, a reduction in the terminal differentiation of ORN is observed [186]. This differentiation defect is associated with a reduction of TGFβ-RII protein levels. These results indicate that interactions between TGF-α and TGF-β signaling pathways are responsible for the correct differentiation of ORN *in vivo* [186].

Section 1.3.5.4 BMP family

In the nervous system, BMP activity is well characterized in the establishment of dorso-ventral patterning [187, 188]. A lot of evidence now indicates that in addition to specifying regional patterning within the developing neural tube, members of the BMP family members can also regulate neuronal and glial differentiation.

Expression and function during neurogenesis

Close to the spinal cord, BMP are produced by the epithelial ectoderm overlying the neural tube and also in the dorsal most part of the spinal cord itself where they play a role in the generation of dorsal cell types [189]. Consistent with this hypothesis, exposure of neural tube explants to BMP4, -5, –7 or related TGF-β members (Dsl1, Activin A or Activin B) induced the generation of spinal cord interneurons [189]. The BMP factors seem also to be important cues to bias progenitor identity toward other specific neuronal fates. For example, BMP9 is a major differentiating factor for cholinergic CNS neurons [190].

BMP can also trigger neuronal differentiation of neocortical precursors from the VZ in different culture systems [132, 191]. This effect on progenitor cells is highly stage and dose dependent. At early stages in the mouse (E13) BMP inhibit proliferation and promote cell death. Later on (E16), they induce neuronal or astroglial differentiation at low doses, but promote cell death at high concentrations [132, 192]. During perinatal development BMP signaling enhances astrogliogenesis and blocks oligondendrocyte differentiation [132]. Repression of BMP activity using its potent natural inhibitor Noggin, which acts by high affinity binding to BMP and prevents BMP binding to cell surface receptors, confirms the activity of BMP in the differentiation of neocortical neurons. Accordingly, Noggin inhibits neuronal differentiation triggered by BMP [193]. Interestingly, Noggin protein expression is detected from E15 in the developing cortex, suggesting a balanced regulation of neocortical neurogenesis mediated by Noggin and BMP interaction *in vivo* [193]. In the adult SVZ the balance between Noggin and BMP also seems to regulate neuronal-glial production but in the opposite manner, suggesting that various progenitor populations are differently sensitive to the BMP-Noggin balance [194]. In this case, Noggin is expressed by ependymal cells adjacent to the SVZ, whereas BMP and their receptors are expressed by SVZ cells. BMP signaling enhances glial differentiation and the counteracting Noggin promotes neuronal differentiation by inhibiting BMP activity [194].

At late developmental stages, and in rostral regions, BMP activity is involved in the generation of astrocytes [188]. Isolated mouse cerebellar granule cell precursors from the outermost proliferative zone of the external germinal layer can differentiate into astroglial cells when exposed to BMP [195]. The astroglial differentiation induced by BMP appears at the expense of oligodendrogenesis and neurogenesis and BMP are in fact potent inhibitors of oligodendrocyte specification, as it has been shown in the chick spinal cord [196]. Exposure of proliferating precursors isolated from fetal striatal SVZ to BMP also induces their differentiation into astrocytes and decreases the proportion of cells differentiating into oligodendrocytes or neurons [197]. Along the same line of evidence, BMP2 exposure of telencephalic neural progenitors in culture also promotes the astrocytic differentiation at the expense of neuronal differentiation [198].

In vivo, overexpression of BMP4 in transgenic mice directs progenitor cells to commit to the astrocytic rather than to the oligodendrocyte lineage [199]. In this study, differentiation of radial glial cells into astrocytes was accelerated, suggesting that radial glial cells are a source of at least some of the supernumerary astrocytes.

The early requirement of BMP signaling during embryogenesis has so far impaired the establishment of direct genetic evidence for BMP function *in vivo*. BMP2 and BMP4 mutant mice die before the majority of neural development has occurred [200]. The mouse mutants for either BMPRIA or BMPRII also arrest early in development not allowing, as well, any conclusion about the function of the BMP pathway during neurogenesis [201, 202].

Effectors of BMP pathway

It has been shown that two genes induced by BMP could mediate the apoptosis observed in response to BMP. These two putative downstream mediators are the transcription factor msx2 and the cell cycle regulator p21 (CIP1/WAF1). The inhibition of their induction blocks BMP-induced apoptosis, however, they are not sufficient to induce apoptosis on their own [203].

The role of BMP signaling in the choice of astrocytic cell fate has been associated with the upregulation of some negative HLH factors: Id1, Id3, and Hes5. The results show that HLH proteins could mediate the BMP2-dependence of the neurogenic fate of these cells [198].

Cross talk between signaling pathways

Besides the direct binding of noggin, chordin, and follistatin (BMP antagonists) that modulates BMP activity, other extracellular factors can also influence the BMP pathway by converging and interfering with downstream effectors of the pathway. EGF and FGF, for instance, modulate the BMP pathway by altering Smad protein activity, a major effector of the BMP pathway, thereby achieving differential activities [204-206]. As already mentioned BMP also exerts opposing action to Wnt activity and to Shh in the spinal cord.

TGF-β superfamily members play various roles during neural differentiation. The effects that are generated in their presence seem to be highly dependent on the environment and, therefore, highly modulated spatially and temporally. Contrary to the factors described so far, they don't have mitogenic activity. They are mostly involved in cell fate decisions and could act as instructive or permissive signals to bias the cellular identity adopted by progenitors.

Section 1.3.6 Other factors

Other signaling molecules and growth factors present interesting expression pattern and activity, which could be associated with a role in establishing the neurogenesis gradient during development. The information still fragmentary about the effects of such factors is mentioned in this section.

Section 1.3.6.1 Neurotrophins

Neurotrophins have been involved in neuronal maturation, mostly as survival factors. This activity is described in the neurotrophic hypothesis, which suggests that neuronal survival is tightly linked to the limited amount of neurotrophic factors the neuron encounters when the axon reaches its target [207]. This late developmental role in neuronal maturation supplements the earlier functions during neurogenesis, which are of interest for this chapter.

Ligands, receptors and expression during neurogenesis

Brain-derived neurotrophic factor (BDNF), the nerve growth factors (NGF) and neurotrophins 3 and 4/5 (NT3/4), commonly named neurotrophins are secreted ligands that bind the receptor tyrosine kinases of high affinity TrkA (NGF receptor), TrkB (BDNF and NT4/5 receptor) and TrkC (NT3 receptor). The members of the neurotrophin family also bind to the low affinity receptor $p75^{NTR}$.

Despite having a late developmental role in neuronal survival and axonal pathfinding, the neurotrophins could also be involved in earlier processes of neuronal differentiation. This is notably suggested by the expression pattern of these factors, as well as their receptors,

at early developmental stages. For example, NT3 and its receptor are detected in the developing cortex when neurogenesis is starting [208]. In addition, after plating mouse cortical stem cells, the presence of FGF2, BDNF, NT3 and the receptors (TrkB and TrkC) is detected in the culture [209].

Function in neurogenesis

While in the PNS NT3 is a powerful mitogen, no such evidence has been found for a similar role in the CNS. Instead, *in vitro* experiments suggest that NT3 is able to promote neuronal differentiation [141]. Indeed, while FGF2 treatment of cortical stem cell cultures stimulates progenitor proliferation, the addition of anti-NT3 antibody reduces the number of generated neurons without directly affecting proliferation. Other pieces of evidence suggest that *in vitro*, NT3 and BDNF can induce neuronal differentiation of hippocampal cells in culture [210]. In another set of experiments, NGF in combination with FGF2 has been involved in the activation of proliferation of precursor cells derived from the rat striatum [211].

In vivo analysis of the expression of BDNF and its receptor TrkB suggests a role in optic differentiation. Both messengers are found to be highly expressed in the retinal neuroepithelium, and disruption of BDNF signaling by dominant-negative receptors significantly blocks the normal differentiation of neurons. Two hypotheses have been developed: BDNF could have a specific survival function for the differentiated neurons, or it could be involved more directly in the progress of differentiation [212].

Effectors of the neurotrophin pathway

Neurotrophins are able to stimulate a number of well-characterized intracellular pathways likely to transduce the signal to numerous cellular functions [213]. For example, upon *in vitro* stimulation with BDNF, NT3 or NT4, phosphorylation of MAP kinases is detected, indicating an effective activation of the neurotrophin receptor [209].

At least two studies suggest that the neurotrophin pathway is connected to regulation of proneural activity. The first observation concerns the regulation of Hes1 by NGF in PC12 cells. When treated with NGF, PC12 cells express neuronal markers and start to extend neurites, and a similar effect has been observed when Hes1 activity was blocked using a

dominant-negative form [214]. In order to find the link between the two phenomena, the authors observed that exposing the cells to NGF leads to phosphorylation of the DNA-binding site of Hes1, thereby rendering Hes1 inactive. Moreover, overexpression of Hes1 blocks NGF activity, suggesting that neuronal differentiation induced by NGF requires the inhibition of Hes1 activity [214]. The second observation concerns the induction of the proneural genes *Mash1* and *Math1* in NSC culture stimulated with NGF, BDNF or NT3 [215]. The combination of a mitotic signal (FGF2) with neurotrophins can induce *Math1* or *Mash1* expression but the cells could not differentiate until the mitogenic factor was removed [215], revealing that the sequence of action of different factors could be important for the proper regulation of neurogenesis.

Section 1.3.6.2 Neurokines: LIF, CNTF

Ciliary neurotrophic factor (CNTF) and Leukocyte Inhibitory Factor (LIF) are two cytokines that act via the glycoprotein 130-linked receptor, LIFRb and CNTFRa, the last being specific for CNTF. They have been shown to have pleiotropic actions on different cell types. In the developing CNS, they promote differentiation or survival of astrocytes, oligodendrocytes or neurons but they also act earlier during development to stimulate the renewal of the NSC. One of the first cues for such a role is suggested by the early expression of CNTFRa in the proliferative region of the telencephalon at E14 [216].

In vitro, CNTF/LIF signaling through LIFR activation has been involved in the maintenance of the NSC proliferation coming from embryonic and adult regions undergoing neurogenesis [217].

The mechanism mediating the cytokine action on NSC could require Notch signaling. Indeed, addition of CNTF to forebrain NSC culture can increase Notch1 expression and Notch1 processing [216].

Section 1.3.6.3 PDGF

Concerning PDGF, ligands and receptors are expressed in cortical NSC suggesting that it can be a good candidate for the control of neurogenesis. Moreover, activated receptors

are also detected in NSC indicating that signaling effectively takes place [218]. In addition, a short exposure of NSC culture to PDGF stimulates neuronal differentiation.

Section 1.3.6.4 GH

GH could also be required as a neurogenic factor. In a study of the intracellular regulator of cytokine signaling SOCS2 (an inhibitor of JAK/STAT signaling), which is also a mediator of GH signaling, effects on GH activity and neuronal development have been correlated [219]. SOCS2 is expressed in neuroepithelial cells, and the $Socs2^{-/-}$ mutant brains have fewer neurons than wild type brains suggesting a requirement for this protein during normal neuronal differentiation. In addition to the brain phenotype, there is a reduction in the number of Ngn1 expressing cells. These effects can also be correlated to the deregulation of GH activity: SOCS2 being normally involved in the inhibition of STAT5 activation triggered by GH.

To conclude, many extrinsic factors are required to finely tune nervous system development. Most of them act locally providing mitogenic or differentiating cues to neural progenitors, and at the same time, they can also diffuse positional information to bias neural fate. The neurogenic switch cannot clearly be attributed to a regulation by one or the other factor. It seems, therefore, that regional information is prevalent to a consensus model about the neurogenic switch.

Section 1.4 Cell cycle regulation and neuronal fate determination

Neurogenesis is defined by the acquisition of neuronal features, one of which is the postmitotic state. The transition from a proliferative state to a quiescent mitotic state is therefore a highly regulated process concomitant with neurogenesis.

The maintenance of proliferation in the NSC pool is crucial for the establishment of neural diversity. Understanding the fluctuation of the NSC cell cycle provides, therefore, the basis toward understanding the balance between proliferation and differentiation.

During neurogenesis, the relationship between cell cycle regulation and cell fate determination is controlled by a cohort of extra- and intra- cellular signals. The balance between proliferation and differentiation appears to be regulated from both sides. First, various factors known to regulate neurogenesis seem to control the cell cycle. Second, the cell cycle parameters themselves influence neural differentiation. Regulation of cell cycle length appears to be part of the mechanism leading to neuronal production.

Section 1.4.1 Cell cycle of neuroepithelial cells

Section 1.4.1.1 Interkinetic nuclear migration and cell division

Neural progenitor cells are characterized by their arrangement in a single cell layer and their epithelial characteristic. In vertebrates, from a cell cycle point of view, the nucleus of these cells oscillates between the ventricular and the cortical surface (in the apical-basal plan of cell polarity), in a movement called interkinetic nuclear migration. The nucleus divides on the apical side of the epithelium, then moves basally during G1 phase. In the basal region, S phase occurs and the nucleus returns apically during G2 phase to divide again [220].

In the neuroepithelium three types of cell divisions have been described in respect to the fate of the daughter cells. Symmetric, proliferative divisions generate two progenitors. Differentiative divisions can be subdivided in symmetric and asymmetric neuron-

generating divisions [221-226]. Before neurogenesis, NSC proliferate and increase the pool of NSC by symmetric, proliferative division. At the beginning of neurogenesis, some cells switch to differentiative divisions that generate either a neuron and a NSC, in the case of asymmetric neuron-generating division or two neurons, in the case of asymmetric neuron-generating division. While neurogenesis proceeds, the number of differentiative divisions increases.

Section 1.4.1.2 Cell cycle length of neural stem cells

Cell cycle length during neurogenesis

The first correlation between a change in cell cycle parameters and mammalian neurogenesis dates from the 90s when the group of Caviness analyzed the cell cycle of neuroepithelial cells using the method of cumulative bromodeoxyuridine (BrdU) labeling. Neuroepithelial cells have been found to increase their average cell cycle length in temporal and spatial correlation with neurogenesis [227, 228]. At the onset of neocortical neurogenesis (E11), when the vast majority of neuroepithelial cells undergo proliferative divisions, the average cell cycle length is about 8 hours. Ongoing with the development of the CNS, the mean cell cycle length of neuroepithelial cells constantly increases to reach 18 hours at the end of the neurogenic interval (E16), when most of them have switched from proliferative to neuron-generating cell divisions [227]. This increase of cell cycle length is selectively due to a lengthening of the G1 phase of the cell cycle, whereas the length of the other phases stays constant i.e. about 4 and 2 hours for the S and M phase, respectively. The length of the G1 phase of neuroepithelial cells quadruplicates: from about 3 hours, prior to the onset of neurogenesis, to more than 12 hours at the end of the neurogenic interval [227].

The cell cycle of subpopulations of progenitors

By taking a closer look at what happens in the neuroepithelium at a fixed time point, contradictory studies report homogeneous or heterogeneous cell cycle length. The issue of cell cycle homogeneity of NSC in the VZ is therefore quite controversial.

Despite the supposed presence of different population of progenitors, measures of cell cycle length in the mouse neocortex have suggested that the cell cycle was homogeneous

[229]. However, other studies suggest that a difference in cell cycle length could, in fact, exists. For example, differences in BrdU incorporation have been observed between distinct coexisting subpopulations of radial glial cells, suggesting that if not length, at least some parameters of the cell cycle vary between radial glial cell subpopulations [230]. This discrepancy could, in part, be attributed to the lack, in the first study, of direct markers allowing the discrimination of various subpopulations. Analysis of the cell cycle length of proliferative dividing cells and neuron-generating cells in the neuroepithelium has shown that these two coexisting cell populations have different cell cycle lengths [226, 231]. The neuron-generating cells have a significantly longer cell cycle compared to the proliferating neuroepithelial cells.

Section 1.4.2 Cell fate determinants influencing the cell cycle

Extrinsic and intrinsic factors involved in the determination and progression of neurogenesis act in part through the control of some important cell cycle regulators (for review [232] [233] [234]). Most if not all these regulators of neurogenesis seem to act on the cell cycle either directly or indirectly (see sections 1.1 and 1.2).

Section 1.4.2.1 Extrinsic cell fate determinants regulate the cell cycle

Shh, for example, is a well-characterized morphogen, which is involved in neuronal patterning and differentiation. It has also been shown to act as a potent mitogen affecting cell cycle progression. Using the GAL4/UAS system, inducible expression of Shh in mice inhibits the generation of postmitotic neurons while it increases the proliferation rate of neuroepithelial cells [108]. Similar results have also been obtained in the developing cerebellum. In this case, synthesis and secretion of Shh by Purkinje cells inhibits differentiation of granule cells. Again, inhibition of differentiation has been associated with an increase in cell proliferation [105]. The exact molecular mechanisms by which this cell fate determinant is able to influence the cell cycle progression are not yet clear. Some data suggest, however, that Shh could directly regulate certain cell cycle regulators. Genomic screens have recently shown that activation of the Shh signaling pathway leads to the increase of cell cycle regulators such as cyclinD and N-myc [235].

Another example concerns the Delta-Notch signaling pathway involved in cell fate determination (see section 1.1.1.2). In addition to its activity in the switch between proliferation and differentiation of NSC, the Delta-Notch pathway influences cell cycle progression. Activation of the Notch receptor by Delta is alone sufficient to inhibit neuronal differentiation and, conversely, its repression promotes it. Interestingly, upregulation and down-regulation of the Notch pathway have been shown to increase and inhibit NSC proliferation respectively [236, 237]. This regulation of cell proliferation seems to be played during the G1-to-S transition by the control of p21 and p27 expression, two G1 cell cycle inhibitors [238].

In vitro analyses of the effect of bFGF and Neurotrophin3 (NT3) shows that the cell cycle length correlates, and may determine, differentiation [239]. Dissociated mouse cortical progenitors submitted to bFGF or NT3 exposure proliferate and differentiate respectively. This effect was correlated to shortening and increasing the length of G1 phase respectively.

Section 1.4.2.2 Intrinsic cell fate determinants regulate the cell cycle

Some transcription factors have also been involved in the cell fate decision and cell cycle regulation. The first example concerns proneural genes, which could directly regulate the transcription of some cell cycle inhibitors. Their overexpression in cell culture is effectively associated with an increase of *p27* expression and followed by cell cycle exit [17].

In another example, overexpression of the Phox2b transcription factor in the vertebrate neural tube is both necessary and sufficient to induce neuronal differentiation (probably via the regulation of proneural genes) and, to inhibit cell proliferation [240, 241]. The mechanisms by which Phox2b influences cell cycle progression has not yet been analyzed and, in particular, it is not known if its action occurs during a particular phase of the cell cycle.

Many other examples could be added (see section 1.1 and [232-234]) either as extracellular factors or intracellular factors influencing the cell cycle. The few studies reported here provide some typical examples about cell cycle regulations. The preceding

examples suggest that the coordination of the cell differentiation program and cell cycle is dependent upon cell fate determinants, which can influence directly or indirectly cell cycle regulators [232, 242]. This view considers the inhibition of cell cycle progression as a consequence of a cell fate change and many studies seem to support this hypothesis.

Besides the dominant idea that cell fate determinants are upstream of cell cycle regulation, emerging data argues that cell cycle parameters by themselves could influence cell fate.

Section 1.4.3 Cell cycle regulators influencing cell fate

Section 1.4.3.1 Cell cycle regulators

The main group of cell cycle regulators is the cyclin dependent kinases (CDK) family. The activity of various members of the CDK family constitute the “thermostat” of cell cycle progression. The general principle is that activation of CDK leads to, and is essential for, progression through the cell cycle. An increase of CDK activity leads to an increase in cell proliferation and, conversely, the inactivation of CDK lengthens, or even blocks, cell cycle progression.

The activity of CDK is essentially regulated by three mechanisms. First, CDK are activated after binding with their respective cyclin partners, whose synthesis and degradation are tightly controlled. Then, they are activated through phosphorylation on threonine residues by the CDK activating kinases. Finally, the cyclin/CDK complexes are inhibited via interaction with the CDK inhibitors (CKI) such as p16, p21 and p27 (for comprehensive review see: [243, 244]).

Section 1.4.3.2 Cell cycle regulators regulate cell fate determination

Manipulating the activity of cell cycle regulators involved in the control of G1 progression, either *in vitro* or *in vivo,* triggers not only changes in cell cycle parameters, but also cell fate changes of neuronal precursors [232-234, 242].

One significant report in this direction concerns the proliferation arrest of pheochromocitoma cells (PC12) and the acquisition of a postmitotic neuronal phenotype

under NGF exposure. This change in cell fate has been correlated with inhibition of CDK2 activity. Interestingly, when CDK2 is directly inhibited, by antisense probes or by treatment with the specific CDK2 inhibitor olomoucine, PC12 cells acquire a neuronal phenotype independent of NGF stimulation [245]. Another *in vitro* example of the influence of cell cycle regulators on the cell fate is given by the overexpression of p73, a known inhibitor of CDK activity. Similarly to PC12 cells, which can be activated to acquire a neuronal phenotype by NGF, retinoblastoma cells can be induced to differentiate by RA. In retinoblastoma cells treated with RA, neuronal differentiation is associated with upregulation of p73 expression. Overexpression of p73 and the consequent inhibition of cell cycle progression is alone sufficient to induce neuronal differentiation in the absence of RA [246].

The activity of p27 (another CKI) has also been implicated in neural fate determination. In both neuronal and glial progenitor cells, the cytoplasmic concentration of p27 increases over time. The “intrinsic timer” model proposes that gradual accumulation of p27 after each cell cycle constitutes the core of the clock, thereby controlling the timing of differentiation. Reaching the appropriate concentration of p27 would eventually lead to a cell cycle block of progenitor cells and their differentiation [247-249]. However, in oligodenrocyte differentiation, the effect of p27 on cell fate determination has been shown to be independent of its capability to control cell cycle [250]. Moreover, inducible overexpression of p27 in the neuroepithelium of transgenic mouse embryos seems to increase neuronal production without visible effects on the cell cycle length of neuroepithelial cells [251].

In line with these observations, *in vivo* analyses strengthen the role of cell cycle regulators in the differentiation process. Indirect evidence suggests that slowing down the cycle induces neuronal differentiation during mouse neurogenesis. Expression of the anti-proliferative gene *TIS21* during G1 phase [252, 253] correlates with neurogenesis and is confined to a subpopulation of neuron-generating neuroepithelial cells [226, 254]. The presence of TIS21 before the differentiative division suggests indeed that cell cycle inhibition happens before the progenitor exits the cell cycle. As more direct evidence, the overexpression of PC3 (the rat homolog of TIS21) is alone sufficient to enhance

neurogenesis, inhibiting at the same time the extent of neuroepithelial cell proliferation [255]. In agreement with the previous observations, the inhibition of the cell cycle progression by olomoucine is alone sufficient to trigger premature neurogenesis in mouse embryos developing in whole embryo culture [256].

As described in this section, the inhibition of cell proliferation may lead to cell fate change. So far it has often been considered that blocking the cell cycle was the cause of differentiation [248]. However, *in vivo* data suggest that lengthening rather than blocking the cell cycle is upstream of cell differentiation.

Section 1.4.4 Cell cycle lengthening model

Considering the proliferation to differentiation switch as the cell fate change of interest, the regulation of cell cycle kinetics has been shown to be essential. The crucial phase of the cell cycle involved is G1 phase. In all cases reported, shortening of G1 phase has been associated with inhibition of differentiation, while inhibition of G1 progression correlates with stimulation of neurogenesis. G1 phase lengthening, which occurs in neuronal progenitors is an upstream event preceding neuronal differentiation.

The lengthening model proposes that an increase in length of the critical phase of the cell cycle leads to differentiation. The underlying mechanism postulated by this model is that a critical activity dependent upon time triggers a cell fate change. To illustrate this model, the regulation of activity of a critical cell fate determinant could require its accumulation or its degradation. For example, a factor specifically synthesized during G1 phase and whose activity is dependent upon a certain threshold would therefore be dependent on the length of the G1 phase to be effective. In other words, a short cell cycle would not allow the cell fate determinant-mediated effects, thus preventing cell fate change.

With regard to the asymmetric distribution of some cell fate determinants during mitosis (see section 1.4), this model would explain how an asymmetric distribution of cell fate determinants would or would not lead to an asymmetric cell fate of the daughter cells. This would be consistent with the observation that lengthening of the cell cycle occurring at the onset of neurogenesis correlates with asymmetric neuron-generating cell divisions

whereas a further lengthening, occurring at later stages of neurogenesis, correlates with symmetric neuron-generating cell divisions [223, 227, 257].

Section 1.5 Neuron-generating asymmetric cell division

Section 1.5.1 Background

Observations of the NSC and their progeny in mammals have indicated the existence of two kinds of divisions: the symmetric, proliferating division generating two NSC and the asymmetric, neuron-generating division giving rise to one neuron and one self-renewing progenitor. This discrimination has been based on the cell fate of the progeny.

The past twenty years have seen the emergence and the characterization of the mechanisms underlying the symmetry versus asymmetry of cell fates. The mechanisms involved seem mostly to be intrinsic to the cell lineage and concern the symmetry of inheritance of fate determinants by the daughter cells. This inheritance is crucial to bias the identity of the receiving cell.

Asymmetric cell division can hence be defined on a cell biological basis or on a cell fate basis. In the first case, the asymmetry of division can already be identified in the mitotic mother cell while in the second case the analysis of the daughter cells is required.

Cell fate asymmetry has been studied in the nervous system of diverse organisms, as an essential mechanism to regulate the generation of neurons from NSC. In *Drosophila*, the mechanisms under this fate asymmetry have been extensively analyzed providing the evidence for the determinant-distribution asymmetry model. In mammals, some data has emerged pointing to the existence of similar mechanisms involved in the establishment of cell fate asymmetry. In parallel, the rising progress in live-imaging has produced many descriptive studies on cell lineages, and in particular the mammalian neural progenitor lineage, providing a more detailed characterization of cell fate asymmetry.

Section 1.5.2 Asymmetric cell divisions of neuroblasts in the *Drosophila* CNS

Section 1.5.2.1 Cell lineage

Strong genetic tools used in *Drosophila* have allowed researchers to analyze the molecular mechanisms of the asymmetric cell division involved in the generation of neurons in the CNS. The molecular machinery have been first identified in the neurogenesis of *Drosophila* and have later been found in other developmental model.

In the *Drosophila* CNS, neurons arise from neuronal precursors called neuroblasts (NB) arranged in a polarized neuroectodermal epithelium. As neurogenesis starts, selected NB leave the epithelium and migrate to a basal position in a process called delamination. Shortly after delamination, NB divide asymmetrically along the apical-basal axis of cell polarity [258]. The daughter cell, which is closer to the apical surface remains NB, while the other daughter (the closest to the basal side) becomes a small ganglion mother cell (GMC). NB division can be considered as a stem cell like division, as the produced NB continue to divide, while GMC divide only once more to produce a pair of neurons or glial cells [259, 260]. In *Drosophila*, all neuronal and glial lineages arising from NB have been traced and identified [258, 261-263].

Section 1.5.2.2 Apical-basal NB polarity

The molecular machinery involved in NB asymmetric division have been highly analyzed. The maintenance of apical-basal cell polarity and the tight control of spindle orientation during division constitute a prerequisite for the asymmetric cell division of NB (for review see [264, 265]).

Prior to delamination, NB are parts of the neuroectodermal epithelium, and similar to the neuroectodermal cells, they are characterized by epithelial polarity features. They are notably connected to the adjacent cells by the junctional complex of the zonula adherens. When NB delaminate (in a basal movement), they loose their cell contacts and move inside the embryo. NB translocation is accompanied by a loss of the apical stalk in the neuroectodermal epithelium and by a rounding up of NB after delamination (see review

[264, 266]). Despite the striking cell shape remodeling, the apical-basal polarity is kept during the delamination process.

The maintenance of polarity, that constitutes an essential step in asymmetric division, has been attributed to a well-conserved complex of proteins: the PAR-6/PAR-3/aPKC (atypical Protein Kinase C) complex. This complex was originally characterized in *C. elegans,* in studies concerning the establishment of the zygote cell division and polarity. In the zygote, the distribution of the Par/aPKC protein complex defines the anterior domain of the cell cortex, and is indispensable for asymmetric division (for review see [267, 268]). Homologs of these proteins have also been found in *Drosophila*. They are located in the subapical region of the neuroectoderm, in the apical stalk of NB during delamination, and in the apical cell cortex after NB have fully delaminated. As in *C. elegans*, mutant phenotypes of the genes encoding *bazooka* (*Drosophila PAR-3* homolog), *Drosophila aPKC* and *DmPAR-6* lead to a loss of apical-basal polarity in epithelial cells and in NB. The PAR/aPKC complex seems, therefore, to be a key component of cell polarity which has been conserved between species and used to establish the polarity of various cell types (see review [264, 268, 269]).

Section 1.5.2.3 Control of spindle orientation

Given that both *Drosophila* neuroectodermal cells, that divide symmetrically and NB that divide asymmetrically use a related molecular mechanism to keep their cellular polarity, the distinction between symmetric and asymmetric division should require additional mechanisms. One prominent difference between neuroectodermal and NB cell division is the orientation of their plane of division. Neuroectodermal cell divisions occur in the plane of the neuroectoderm while NB divisions occur in the apical-basal axis. The orientation of the mitotic spindle constitutes, therefore, a crucial step to generate the asymmetric distribution of factors segregated in the apical-basal axis.

The protein Inscuteable is the major player of this process. Inscuteable is not expressed in neuroectodermal cells, and the protein is first detected in the apical stalk of NB during delamination. In delaminated NB, the localization of Inscuteable to the apical cell cortex is reestablished at each cell cycle between late interphase and anaphase [266]. In absence

of the protein, the mitotic spindle in NB fails to rotate [270], and NB divide in random orientations [271]. The connection between apical-basal polarity and mitotic spindle orientation is established via the protein Bazooka that is required for apical localization of Inscuteable [272, 273]. Inscuteable recruits Pins (Partner of Inscuteable) and the associated G protein αi subunit. The apical colocalization of Inscuteable, Pins, and Gαi is interdependent and essential for proper asymmetric cell division. So far, however, the mechanism underlying the regulation of spindle orientation by the complex Inscuteable/Pins/Gαi is not clear, and no direct interactions have been found with microtubules or the centrosome that would account for the activity of the Inscuteable/Pins/Gαi complex [264].

Section 1.5.2.4 Cell fate determinants

Cell fate determinants are mostly proteins, Prospero, Miranda, Staufen, Numb and Partner of Numb (PON) but also mRNA (*Prospero*). These determinants are localized in a basal crescent in NB, and inherited by the GMC. Miranda, Staufen and PON are adaptor proteins required to recruit Prospero (protein and mRNA) and Numb at the basal cell cortex. Miranda binds to Prospero and Staufen [274, 275]. Staufen, an RNA binding protein, segregates Prospero mRNA by binding to its 3' untranslated region [276, 277]. Prospero, a homeobox transcription factor, is required for the transcription of GMC specific genes but also for the down-regulation of NB specific genes in the GMC [278, 279]. Numb is transported to the GMC during metaphase by PON's activity [280]. Numb acts by repressing Notch activity in the GMC, however, the mechanisms of Numb activity are not yet clear [265, 268, 281].

The tight localization of the protein complexes described so far is crucial for the asymmetric segregation of cell fate determinants. For example, the apical protein Inscuteable is required for the proper localization of Numb, Prospero, and Miranda. All three proteins still localize asymmetrically without Inscuteable, but their crescents are formed at random positions around the cell cortex and are no longer correlated with the spindle poles [268, 270]. In addition to the apical complexes, the protein products of the tumor suppressor genes *lethal giant larvae* (*lgl*), *discs large* (*dlg*) and *scribble* (*scrib*) are essential for the basal localization of cell fate determinants [282, 283]. While the

distribution of these proteins is not asymmetric, the activity of Lgl is nonetheless restricted to the basal cortex by phosphorylation triggered by the apical DaPKC protein [284].

Section 1.5.3 The neuronal cell lineage in the mammalian CNS

Section 1.5.3.1 Neuron-generating divisions and cell fate asymmetry

The pool of neural progenitors that constitutes the wall of the neural tube is exponentially amplified by symmetric, proliferative division before neurogenesis. When neurogenesis starts, the neural progenitors switch from a symmetric to an asymmetric mode of division. After neurogenesis, an identity switch towards glial cell type differentiation takes place. It is assumed that the completion of neural differentiation is characterized by another switch from an asymmetric to a symmetric mode of division giving rise to two differentiated cells. However, this simplistic model has been reevaluated by the analysis of clonal progeny of mammalian NSC (*in vitro* and *in vivo*). It has been shown that neurons come from two coexisting type of divisions: asymmetric neuron-generating divisions and symmetric neuron-generating divisions, which generate two neurons [221, 224-226].

In the mammalian nervous system, the fate asymmetry resulting from the asymmetric division of neuronal progenitors has been traditionally deduced from lineage studies. Analyses using single labeled neuroepithelial cells *in vivo* coupled to characterization of the progeny (morphology and localization) have established the first basis for the progenitor versus neuron fate asymmetry of the daughter cells [224, 285, 286].

Recent progress in imaging and particularly time-lapse recording systems in intact living tissue have brought convincing evidence and numerous information about distinct daughter fates. According to this strategy, time-lapse studies of dividing neuroepithelial cells or radial glia cells (which can be considered as a subpopulation of neuroepithelial cells, see [264, 287]) have been followed in slice cultures of mammalian embryonic brains. Asymmetric morphology, marker localization (as for static analysis), and asymmetric behavior of the daughter cells have been taken as evidence for an

asymmetric, neuronal versus progenitor, fate. Kriegstein and colleagues followed GFP labeled precursor cells and their progeny *in vivo* up to 3 days. They showed that the radial glial cell is indeed a neuronal precursor. In addition, radial glial cells maintain their pial processes (also called the “basal process”) throughout cell division, although it becomes extremely thin at certain time points of mitosis [288]. Similar results have been reported after examination of the morphology and the cell cycle of differently labeled radial glial cells (dye labeling from the pial surface in a cortical slice culture) [289]. Up to 140 hours of recording of the embryonic radial glial cells have allowed the description of detailed patterns of cell division and migration [225].

The neuron-generating division has also been specifically recorded and characterized using GFP knock-in mice. In this study, *GFP* has been inserted into the *TIS21* locus, encoding an anti proliferative gene, whose mRNA is selectively expressed in neuron-generating, but not proliferative neuroepithelial cells nor in neurons [226, 254]. Two kinds of neuron-generating divisions have been observed [225, 226]: apical divisions resulting in asymmetric neuron-progenitor daughter fate, and basal divisions resulting in symmetric neuron-neuron daughter fate.

The mechanisms that could explain neuronal fate are still not as well understood as in *Drosophila*. The coexistence of two types of neuron-generating progenitors raises the question of the existence of two different mechanisms involved in neuronal production in the mammalian CNS. The basally dividing progenitor generating two neurons is reminiscent of the GMC in *Drosophila* and suggests that similar factors and asymmetric cell division could be involved in its generation. This would also suggest the existence of NB-like neuroepithelial cells, which are, if they exist, indistinguishable from the proliferative progenitors.

The mechanisms of fate asymmetry resulting from the apical neuron-generating division seem to depend, like in *Drosophila*, upon the segregation of progenitor versus neuronal determinants (see the next section).

Section 1.5.3.2 Cell Polarity in the mammalian neuroepithelium

Asymmetric distribution of some cellular determinants has also been described in mammals. The data collected so far in mammals suggest that, on one hand, some features could be shared with the fly mode of neurogenesis, and on the other hand, distinct mechanisms could be involved.

Like all epithelia, mammalian neuroepithelial cells have an apical-basal polarity. The epithelial characteristics of the neuroepithelial cells change with development. During the neural plate stage, neuroepithelial cells show the typical features of epithelial cells. They are polarized, with distinct apical and basolateral surfaces [290], the polarity is maintained by functional tight junctions [291], and their basal plasma membrane is in contact with a basal lamina [292]. With neural tube closure and the onset of neurogenesis, neuroepithelial cells start to loose some of these epithelial features. They switch from E-cadherin to N-cadherin expression [293], functional tight junctions are lost [291], and the polarized delivery of certain membrane proteins to the apical and basolateral plasma membrane is down-regulated [290].

Section 1.5.3.3 Neuron-generating division and asymmetric cell division in mammals

It has been proposed that the type of division neuroepithelial cells undergo critically depends on the orientation of the cleavage plane relative to the apical-basal cell axis, like for *Drosophila* NB divisions. Vertical cleavage planes (i.e. parallel to the apical-basal axis) would give rise to symmetric, proliferative divisions while horizontal cleavage planes (i.e. perpendicular to the apical-basal axis) would give rise to asymmetric, neuron-generating divisions [294-296]. However, the proportion of horizontal cleavages observed is not enough to be able to account for the neurons produced (particularly during the peak of neurogenesis): there are too few horizontal cleavage planes [83, 297, 298]. It has, therefore, been suggested that neuron-generating as well as proliferative divisions of the neuroepithelial cells could have vertical cleavage planes.

Given the particularly elongated shape of the neuroepithelial cells, the small apical domain of the cell could easily be asymmetrically distributed with a vertical cleavage

plane. Such an unequal partition of the apical plasma membrane to one daughter could lead to an asymmetric distribution of determinants in the small apical region (as it was previously described in *Drosophila*) [299]. To test this hypothesis, the position of the apical plasma membrane (Fig. 4, white box) relative to the cleavage plane (Fig. 4, dotted line) has been examined in proliferative or neuron-generating divisions using the *TIS21*-GFP knock-in to discriminate the two progenitors [226]. The switch from proliferative to neuron-generating divisions correlates with the symmetric versus asymmetric inheritance of the apical membrane rather than with the vertical versus horizontal rotation of the cleavage plane [300]. This result suggests, hence, that some determinants, being in this case the apical plasma membrane, are asymmetrically distributed and correlated to a specific cell fate in mammalian neuron-generating divisions.

Section 1.5.3.4 Asymmetric distribution of cell fate determinants in mammals

So far, very little is known about the mechanisms triggering asymmetric division in mammals. Homologs of the Par3/Par6/DaPKC do exist and their distribution in the apical region (junctional distribution) has been described [301]. Moreover, the mammalian homolog of *Drosophila* Bazooka (mPAR-3) is distributed equally or unequally in symmetric, proliferative or asymmetric, neuron-generating dividing neuroepithelial cells, respectively [300]. mPAR-3, and likely its binding partners mPar6 and aPKC, could therefore be involved in the control of the apical-basal polarity and the symmetry of the cell division as in *Drosophila*. One possible cell fate determinant, mammalian Numb, has been shown to localize to an apical crescent of neuroepithelial cells [302, 303], however, the role of Numb in mammalian neurogenesis still needs to be determined [264, 304].

The comparison between *Drosophila* and mammals is highly suggestive of a strong conservation of the mechanisms involved in neuron generation. Even the apparent contradiction concerning the cleavage plane orientation could find an explanation in the atypical morphology of the mammalian neuroepithelial cells [300]. The comprehension of the mechanism underlying the production of neurons either in *Drosophila* or in mammals represents an important step in the understanding of neurogenesis.

Section 1.6 Conclusions

Spatial and temporal control of neurogenesis needs to be tightly coordinated to allow the proper generation of neurons in the proper place, at the proper time.

Many regulators of neurogenesis are either involved in the promotion of differentiation or in the regulation of proliferation. The neurogenic switch, revealed at the level of the cell cycle division, requires the mobilization of various factors and mechanisms in the neural stem cells. As highlighted in this chapter, some core common mechanisms are involved in neuronal differentiation. Transcriptional regulation, cell cycle control and asymmetric distribution of cell fate determinants are part of these common critical steps involved in the control of neuronal generation. However, a consensus concerning the events upstream of the neurogenic switch does not exist yet. The local regulation of neurogenesis by distinct effectors could prevail over such a consensus.

Extensive cross-regulations between the distinct pathways and mechanisms regulating neurogenesis make it complicated to fully understand the chronology of events triggering neuronal differentiation. Furthermore, the balance between the diverse signals may be as important as their chronology. Finally, local variations in the integration of these signals may provide the flexibility required to build the central nervous system.

LEGENDS

Figure 1 The neural tube

The progenitor cells (dividing NSC) are arranged in a single cell layer facing the lumen of the neural tube. These cells are polarized: the apical side faces the lumen and the basal side faces the cortical layer. During neuronal differentiation, the young neurons escape from the VZ and accumulate in the cortical layer.

Figure 2 Neuronal precursor selection and lateral inhibition

Inside the proneural group, all the cells are equivalent, and have the capacity to become neuronal precursors.

A disequilibrium in the equivalence group perturb the Delta-Notch signaling, some scattered cells start to emerge by repressing their neighbors via the lateral inhibition pathway.

Selected cells adopt the neuronal fate while the surrounding cells adopt the ectodermal identity (in the *Drosophila* PNS) or keep an undifferentiated fate (in the CNS).

Figure 3 Notch signaling

A Notch processing. 1, Notch is cleaved during its processing in the Golgi apparatus and appears in the plasma membrane as a heterodimer. 2, Upon DSL (Delta/Serrate/Lag-2) ligand activation Notch is processed by the TACE/ADAM proteases, which triggers the release of the extracellular domain. 3, Notch intracellular fragment (NIC) is accessible to

γ-secretase activity, is cleaved, released in the cytoplasm and 4, translocates in the nucleus.

B Transcriptional interactions. 1, CSL (CBF1/RBP-Jk/Su(H)/Lag-1) factors recruit co-repressor complexes and repress *Hes* genes. 2, In presence of NIC, the activator complex NIC/CSL/mastermind transactivates Hes target genes. Hes acts at two levels to repress the proneural activity: 3a, Hes repress proneural genes by forming a complex with the corepressor groucho/TLE, and 3b, Hes forms an inactive complex with the bHLH factors. 4, In absence of proneural repression, the neuronal determination program is activated and leads, notably, to *Delta* (*DSL*) upregulation.

Figure 4 Model of apical plasma membranes distribution in proliferative or neuron-generating dividing neuroepithelial (NE) cells in developing mouse embryo.

Orientation of the cleavage plane (dot line) is almost parallel to the apical-basal axis in both case, but the position of apical plasma membrane (bold line) related to the cleavage plane is equal (*left*) or unequal (*right*). In case of neuron-generating asymmetric division, the daughter cell, which inherits the apical plasma membrane will remain a neuroepithelial cell, while the other one (lacking the apical plasma membrane) will be a neuron.

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Fig. 1

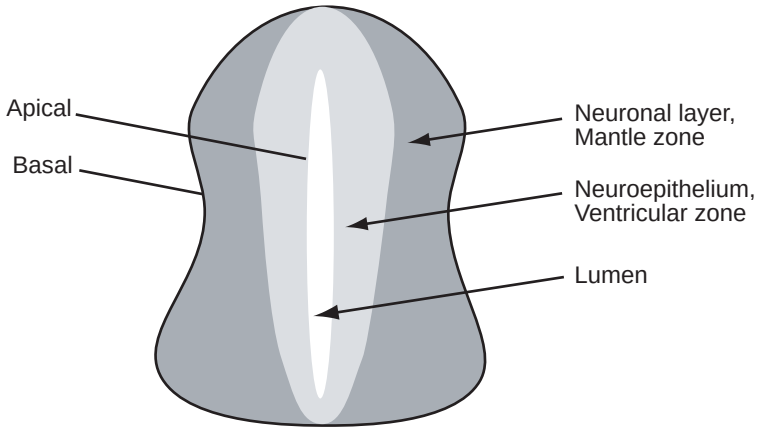

Apical
Basal
Neuronal layer,
Mantle zone
Neuroepithelium,
Ventricular zone
Lumen

Fig. 2

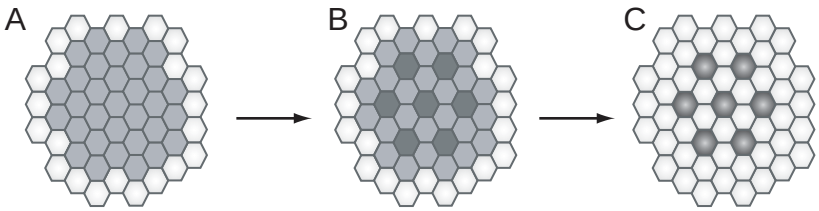

A
B
C

Fig. 3

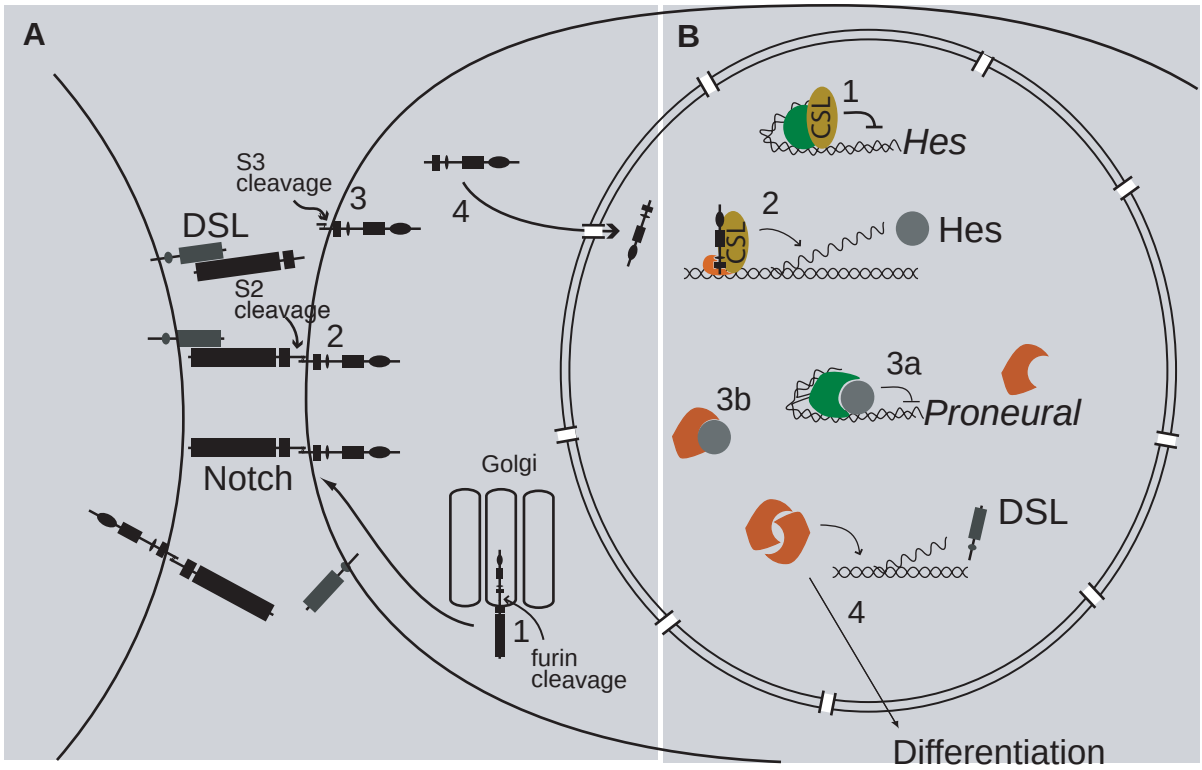
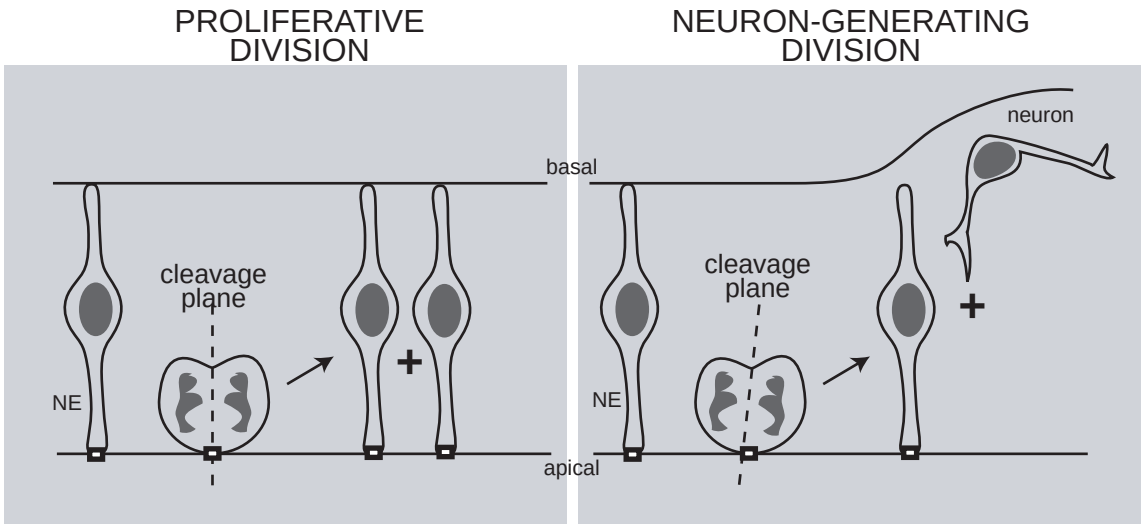

A
B
S3
cleavage
3
DSL
4
S2
cleavage
2
Notch
Golgi
1
furin
cleavage
CSL
1
Hes
2
CSL
Hes
3a
3b
Proneural
DSL
4
Differentiation

Fig.4


PROLIFERATIVE
DIVISION
NEURON-GENERATING
DIVISION
basal
neuron
cleavage
plane
cleavage
plane
NE
NE
+
+
apical